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CAR-T cells dual-target CD123 and NKG2DLs to eradicate AML cells and selectively target immunosuppressive cells

Xin Jin[a,b,c#], Danni Xie[d#], Rui Sun[a#], Wenyi Lu[c], Xia Xiao[c], Yibing Yu[d], Juanxia Meng[c], and Mingfeng Zhao[c]

[a]School of Medicine, Nankai University, Tianjin, China; [b]Liangzhu Laboratory, Zhejiang University Medical Center, Hangzhou, China; [c]Department of Hematology, Tianjin First Central Hospital, School of Medicine, Nankai University, Tianjin, China; [d]First Central Clinical College, Tianjin Medical University, Tianjin, China

ABSTRACT

Chimeric antigen receptor (CAR)-T cells have not made significant progress in the treatment of acute myeloid leukemia (AML) in earlyclinical studies. This lack of progress could be attributed in part to the immunosuppressive microenvironment of AML, such as monocyte-like myeloid-derived suppressor cells (M-MDSCs) and alternatively activated macrophages (M2 cells), which can inhibit the antitumor activity of CAR-T cells. Furthermore, AML cells are usually heterogeneous, and single-target CAR-T cells may not be able to eliminate all AML cells, leading to disease relapse. CD123 and NKG2D ligands (NKG2DLs) are commonly used targets for CAR-T therapy of AML, and M-MDSCs and M2 cells express both antigens. We developed dual-targeted CAR-T (123NL CAR-T) cells targeting CD123 and NKG2DL by various structural optimization screens. Our study reveals that 123NL CAR-T cells eradicate AML cells and selectively target immunosuppressive cells. A highly compact marker/suicide gene, RQR8, which binds targeting epitopes of CD34 and CD20 antigens, was also incorporated in front of the CAR structure. The binding of Rituximab to RQR8 leads to the elimination of 123NL CAR-T cells and cessation of their cytotoxicity. In conclusion, we successfully developed dual effects of 123NL CAR-T cells against tumor cells and immunosuppressive cells, which can avoid target escape and resist the effects of immunosuppressive microenvironment.

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Introduction

Acute myeloid leukemia (AML) is the most common acute leukemia in adults. Standard induction chemotherapy can achieve a complete remission rate of 60–80% in AML. Ultimately, however, 50–70% of AML patients still relapse, with a 5-year survival rate of only 20–30%.[1,2] Chemotherapy resistance and short-term recurrence are still the main influencing factors that threaten the survival of patients.[1,3] With the application of hematopoietic stem cell transplantation and small molecule inhibitors, the survival duration of acute leukemia has been prolonged. However, due to the high recurrence rate, the 5-year survival rate is still low.[4–6] In recent years, chimeric antigen receptor (CAR)-T cells have become an extremely attractive biotechnology in the field of tumor treatment and have been widely used in clinical research. Of the CAR-T cell strategies, CAR-T cells targeting CD19 have shown significant antitumor effects and long-term remission in the treatment of B cell tumors, and BCMA-targeting CAR-T cells have shown promising effects in the treatment of multiple myeloma (MM).[7–9]

Currently, one of the most widely targeted CAR-T cell therapies for AML is CD123, with a large number of clinical trials underway. CD123 is widely expressed (more than 50%) in the original cells of AML patients and is one of the indicators of poor prognosis.[10,11] Compared with normal hematopoietic stem/progenitor cells, CD123 is overexpressed on AML cells.[11] CAR-T cells targeting CD123 have shown extremely high potency in preclinical AML models.[12–17] However, the clinical trial data showed only a modest efficacy.[18–21] NKG2D is highly conserved and has 8 ligands, namely MICA, MICB, and the UL16-binding proteins (ULBP) 1–6.[22] NKG2D ligands (NKG2DLs) are upregulated in response to DNA damage, infection with certain pathogens, and malignant transformation.[23] NKG2DLs expression has been reported in a broad range of solid tumors and hematologic malignancies, including AML and MM, whereas ligands are generally absent in healthy tissues.[24] Thus, NKG2D-based CAR-T cells have the potential for broad oncologic tumor therapy. There are many reports of CAR-T cells targeting NKG2DLs in the treatment of AML, which show good safety, but show the same difficulties as CD123 CAR-T cells, and the efficacy needs to be improved.[23–27]

Previous clinical trial reports have shown that the application of CAR-T cells in AML still faces great challenges.[8,9] The poor persistence, sufficient efficacy of CAR-T cells, and the target escape of tumor cells are several important reasons that hinder the treatment of AML by CAR-T cells. Studies have shown that myeloid-derived suppressor cells (MDSCs) in the tumor microenvironment are associated with insufficient efficacy and poor durability of CAR-T cells.[28–30] It has also been shown that MDSCs, which are abundantly present in the AML tumor

CONTACT Xin Jin jxin29@163.com; Mingfeng Zhao mingfengzhao@sina.com Department of Hematology, Tianjin First Central Hospital, School of Medicine, Nankai University, No. 24 Fukang Road, Nankai District, Tianjin 300192, China

[#]Xin Jin, Danni Xie and Rui Sun contributed equally to this work.

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microenvironment, play a key role in evading immune surveillance.[31,32] Reports have shown that NKG2DLs CAR-T cells can significantly kill MDSCs in the tumor microenvironment of liver tumors and significantly extend the survival time of mice.[33] There are also reports showing that CAR-T cells targeting CD123 can significantly kill M2 cells in the tumor microenvironment in Hodgkin's lymphoma.[34] These data indicate that CAR-T cells targeting NKG2DLs and CD123 may have the potential to act on immunosuppressive cells around tumors in the treatment of AML. One of the conventional methods to overcome the lack of efficacy and target escape is to design CARs that target two cell membrane molecules, called dual-target CAR-T cells.[35,36] Compared with single-target CAR-T cells, dual-target CAR-T cells show stronger activity and affinity for tumor cells.[14,37–39] Previous studies have confirmed that dual-target CAR-T targeting CD19 and CD22 in treating B cell acute lymphoblastic leukemia (B-ALL) and dual-target CAR-T targeting CD33 and CLL-1 in the treatment of AML, both improve the remission rate and have acceptable toxicity.[35,40] These results may indicate that dual-target CAR-T cells have better efficacy than single-target CAR-T cells while avoiding target escape.

In this work, to overcome the problems of target escape and weak durability, and insufficient efficacy of CAR-T cells in treating AML, we propose a novel option for the treatment of relapsed/refractory AML: CD123 and NKG2DLs dual-target CAR-T (123NL CAR-T) cells. Our results show that 123NL CAR-T cells can kill tumor cells and rectify the immunosuppressive environment, resulting in a powerful tumor-killing effect.

Materials and methods

Cell lines and primary cells

HEK-293T cells, DAUDI cells, THP1 cells, and HMy2. CIR cells were purchased from ATCC. BJAB cells were purchased from DSMZ. DAUDI cells, THP1 cells, and HMy2. CIR cells were transduced with firefly luciferase to allow in vivo tumor burden imaging. In addition, DAUDI cells overexpressing MICA or CD123 were produced by transfecting DAUDI cells expressing firefly luciferase with lentiviral supernatant containing the corresponding gene sequence. All cell lines except 293T cells were cultured in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum (Biological Industries). 293T cells used for viral production were cultivated in DMEM high glucose medium supplemented with 10% fetal bovine serum. Primary human AML specimens were acquired from the Laboratory of Hematology Department, Tianjin First Central Hospital. And nformed consent was obtained from the patient. This study design was approved by the Ethics Committee of Tianjin First Central Hospital.

Lentiviral vector

The single-chain antibody targeting CD123 is derived from the 7G3 clone, and the sequence targeting NKG2DLs uses the NKG2D extracellular domain. The single-target CAR structure contains CD123-scFv or NKG2D extracellular domain, human 4-1BB or CD28, and CD3ζ signal domain. The plasmid structure also contained a highly compact marker/suicide gene combining target epitopes from both CD34 and CD20 antigens (RQR8) before the CAR structure, which can be labeled with CD34 antibody and can also be used to eliminate CAR-T cells with rituximab.[41] The dual-target CAR structure consists of two single CARs, and the CAR structure and the RQR8 structure are separated by a self-cleaving 2A peptide. The CD19 CAR vector contains scFv FMC63-based targeting domain, CD8-derived hinge, and transmembrane domain, 4-1BB/CD137 costimulatory domain, and CD3-zeta chain intracellular signal domain. All these structures are connected to a lentiviral plasmid driven by the MND promoter.

Cell line murine model and primary murine model

For the cell line murine model, male NSG mice (6–8 weeks old; Sbefer) were injected intravenously with 2×10^6 tumor cells expressing luciferase. After 5 days, 1×10^6 CAR-T cells or uninfected T cells were injected into the mice through the tail vein. To monitor tumor growth, each murine was intraperitoneally injected with 3 mg of D-fluorescein (Sigma) at the designated time point. Ten minutes later, the mice were imaged with an IVIS Lumina II (PerkinElmer) to assess tumor burden. The animal experiment protocol was approved by the Animal Care and Use Committee of Tianjin Medical University.

Statistical analysis

The results were analyzed by GraphPad Prism 8.0 (GraphPad Software). Normally distributed data are represented as the mean ± standard deviation (SD), multiple group comparisons were performed with one-way analysis of variance (ANOVA), nonnormally distributed data are represented as the median and quartile range, and the Kruskal-Wallis test was used. The Kaplan-Meier method and log-rank test were used for survival curve analysis. $P < 0.05$ was considered to indicate statistical significance. Other methods are provided as supplemental material.

Results

The tumor cells and immunosuppressive cells of AML patients express CD123 and NKG2DLs

To demonstrate the feasibility of simultaneously targeting CD123 and NKG2DLs, we collected bone marrow specimens from 16 de novo AML patients and 10 iron-deficiency anemia (IDA) patients. Compared with IDA patients, although the patient's MDSCs did not increase, the M-MDSCs with strong immune suppression increased significantly, but there is no difference in the proportion of M2 cells (Figure 1a, b). Most of the patient tumors, M-MDSCs, and M2 cells expressed CD123. NKG2DLs are expressed in M-MDSCs and M2 cells, however, the expression is low in tumor cells (Figure 1c–e, Fig. S_1a and b, Table S1). Earlier studies have shown that NKG2DLs are under-expressed in primary AML cells, and the addition of IFN-γ can promote its significant up-regulation.[42] Collect serum after 24 hours of CD123 CAR-T and target cells at a ratio of 3 to 1, and co-culture with primary AML cells (The serum has an IFN-γ that is 10

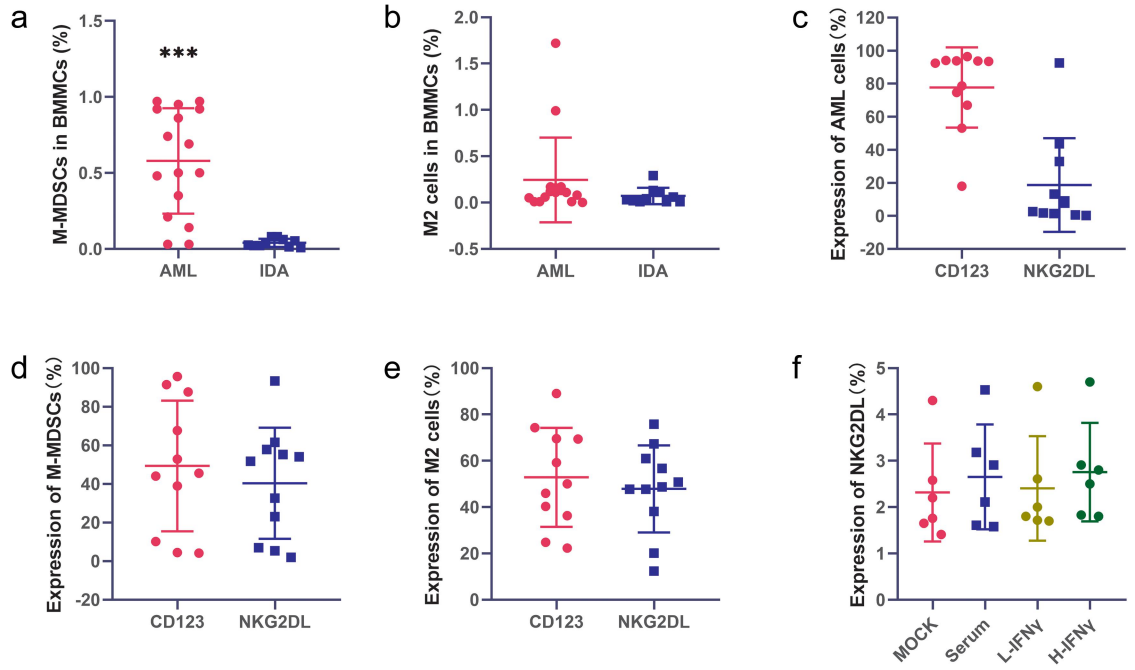

Figure 1. The tumor cells and immunosuppressive cells of AML patients express CD123 and NKG2DLs. **a** the proportions of M-MDSCs in AML patients and IDA patients. **b** the proportions of M2 cells in AML patients and IDA patients. **c** CD123 and NKG2DLs (MICA/MICB, ULBP-1, ULBP-2/5/6, ULBP-3, ULBP-4) expression on the surface of AML tumor cells. **d** CD123 and NKG2DLs expression on the M-MDSCs surface. **e** CD123 and NKG2DLs expression on the M2 cell surface. **f** primary AML cells were cocultured with supernatant from tumors treated with 123 CAR-T cells, a low concentration of IFN-γ recombinant protein, and a high concentration of recombinant IFN-γ for 7 days. Flow cytometry was used to detect the expression of NKG2DLs. Three independent repeated experiments were performed, and the data are expressed as the mean ± standard deviation; ***$p < 0.001$. MOCK refers to a blank control without adding any reagents.

times higher than normal), a low concentration of IFN-γ recombinant protein (1 ng/mL), and a high concentration of recombinant IFN-γ (10 ng/mL). Flow cytometry showed that the expression of NKG2DLs on primary AML cells was not upregulated even when the coculture for more than 7 days (Figure 1f). NKG2DLs CAR-T cells have been demonstrated to be effective for AML patients in clinical studies. We hypothesize that their effectiveness may be attributed to mechanisms other than direct targeted killing.

Optimization of the vector structure of the CD123 and NKG2DLs dual-target CAR

We first designed a series of CARs that individually targeted CD123 or NKG2DLs (Fig. S_2a). Our CAR was expressed on the surface of T cells as shown by IgG(H+L) antibody and NKG2D antibody detection (Fig. S_2b and c). Lymphocyte function-associated antigen 1 (LFA-1) is a key protein for the formation of immune synapses before T cells induce cytotoxicity. It is worth noting that on the ninth day of culture, we found that the NKG2DLs CAR-T cells expanded slowly, and their culture medium had higher levels of cytokines, especially when CD28 was used for the costimulatory domain or transmembrane domain (Fig. S_2 d and f). The use of LFA-1 inhibitors can inhibit the cytotoxicity of T cells. We found that the addition of LAF-1 inhibitor (2.5 nM) to the NKG2DLs CAR-T cell culture medium increased NKG2DLs CAR-T cell expansion and reduced the level of cytotoxicity-related cytokines in the culture medium (Fig. S_2e and f). Further testing revealed that the NKG2DLs CAR-T cells expressed NKG2DLs on the surface (Fig. S_2g). These data suggest that there is fratricide killing of NKG2DLs CAR-T cells, which can be reduced by using 4-1BB as the costimulatory domain and the CD8 hinge structure. Therefore, we did not use the NKG2DLs CAR structure featuring a costimulatory domain or transmembrane domain based on CD28 in the following experiments. To evaluate the antigen recognition ability of the CAR-T cells, we screened the expression of CD123 and NKG2DLs in 20 cell lines preserved in our laboratory (Figure 2a, b). And ultimately 4 cell lines were selected for evaluation: DAUDI cells did not express either antigen, while THP1 cells expressed both antigens. BJAB cells exclusively expressed NKG2DLs, and HMY2.CIR cells solely expressed CD123 (Figure 2c). The single-target CAR-T cells obviously killed tumor cells that were positive for the target antigen and secreted large amounts of cytokines (Figure 2d, e). Next, we designed a series of bicistronic CARs by optimizing the hinge and costimulatory domains in the anti-CD123 CAR structure and called these CARs BCAR1–4 (Figure 3a). These four BCARs were differentially expressed in human peripheral blood T cells infected with lentivirus, indicating that we successfully prepared four different 123NL CAR-T cells (Figure 3b). We then tested the targeted toxicity of these CAR-T cells. BCAR3 showed strong cytotoxicity, and the proportion of cells with a naive phenotype in the CAR-T cells was higher than that seen with the other BCARs, while the level of immune checkpoint molecules was lower (Figure 3c, d, Fig. S_3a-d).

There have been a large number of reports showing that CAR-T cells with a higher proportion of cells with a naive phenotype and lower levels of immune checkpoint

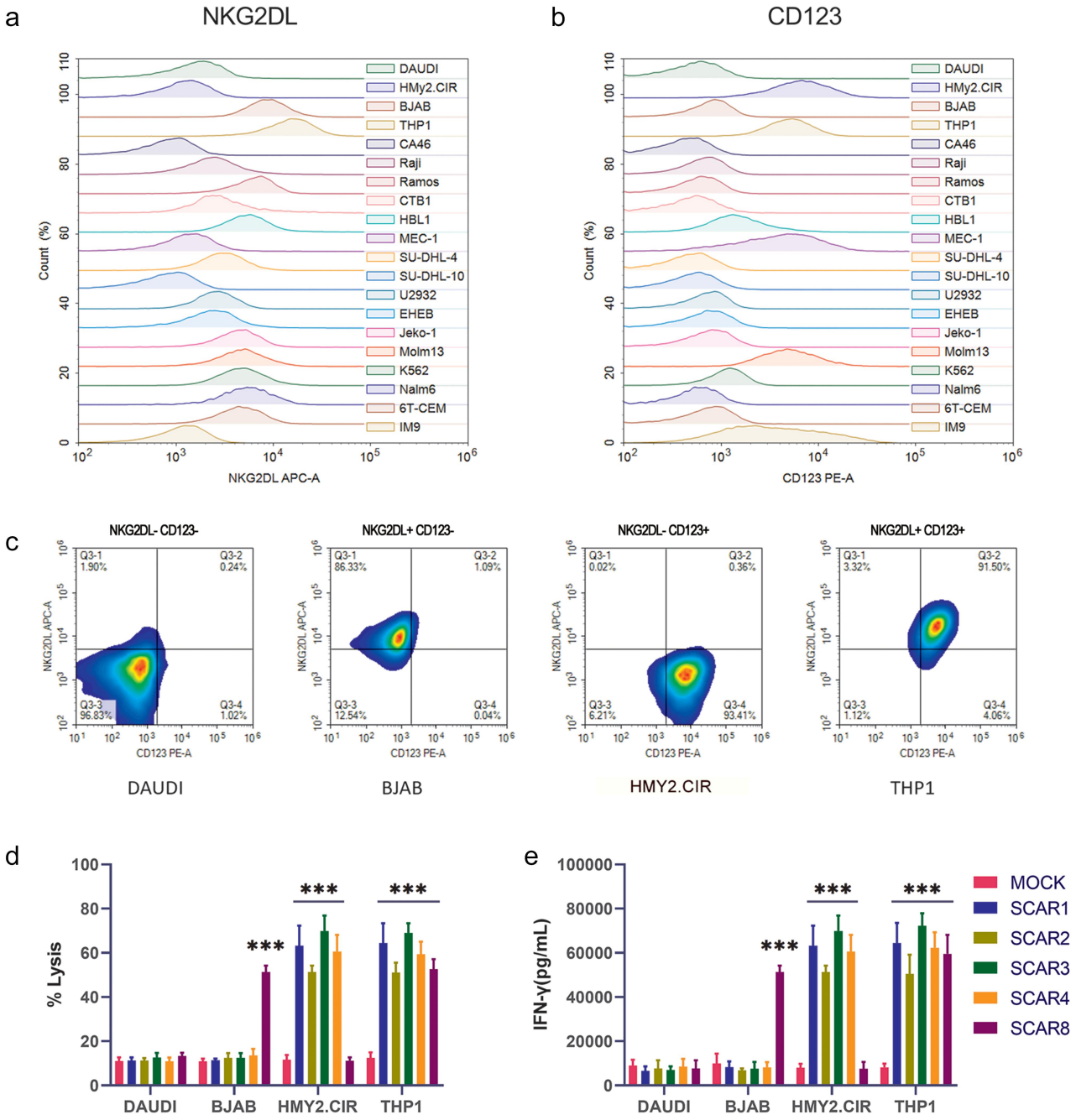

Figure 2. Cell line screening and single-target CAR-T cell function verification. a flow cytometry to detect the expression of NKG2DLs in 20 common hematological tumor cell lines. b flow cytometry to detect the expression of CD123 in 20 common hematological tumor cell lines. c the NKG2DLs and CD123 expression of the four cell lines were as follows: DAUDI cells did not express either marker, THP1 cells expressed both markers BJAB cells expressed only NKG2DLs and HMY2.CIR cells expressed only CD123. d single-target CAR-T cells with various CAR structures were cocultured with DAUDI, BJAB, and HMY2.CIR cells were in equal proportions for 24 hours, and the cytotoxicity of each group was detected. e single-target CAR-T cells with various CAR structures were cocultured with DAUDI, BJAB, and HMY2.CIR cells were in equal proportions for 24 hours, and the level of secreted IFN-γ (a cytotoxicity-related cytokine) in the culture medium was detected. Three independent repeated experiments were performed, and the data are expressed as the mean ± standard deviation; ***$p < 0.001$. MOCK indicates uninfected T cells.

molecules have better persistence and better efficacy.[43–45] Therefore, we used the 123NL CAR-T cells with the BCAR3 structure for the subsequent experiments.

123NL CAR-T cells can kill tumor cells in vitro

To further demonstrate the target specificity of 123NL CAR-T cells with the BCAR3 structure, we induced overexpression of CD123 and MICA (an NKG2D receptor) in DAUDI cells, which do not express CD123 and NKG2DLs. After screening and single-cell clone culture, flow cytometry confirmed that the overexpression cell lines were successfully established (Figure 4a). The selected overexpression cell lines were incubated with 123NL CAR-T cells or uninfected T cells at ratios of 1:1, 1:2, 1:4, and 1:8 and detected by flow cytometry after 48 hours. The cell killing rate was calculated, the supernatant was collected, and secreted cytokines were detected by flow

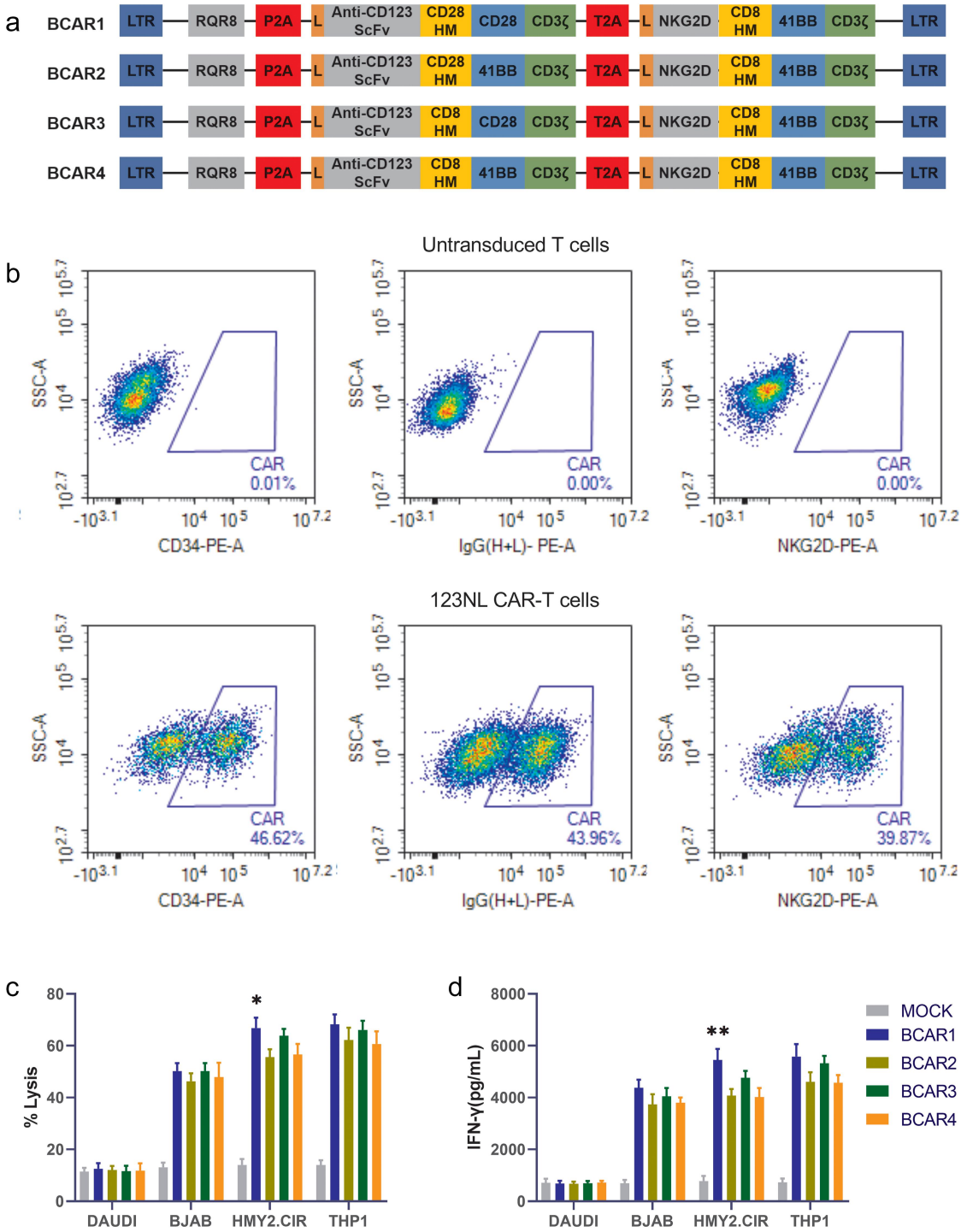

Figure 3. Functional verification of dual-target 123NL CAR-T cells. a diagram showing the structures of the four bicistronic CARs simultaneously targeting CD123 and NKG2DLs. RQR8 is a highly compact marker/suicide gene combining target epitopes from both CD34 and CD20 antigens. L is the signal peptide, HM is the hinge and transmembrane structure, and T2A and P2A are the split peptides. b Representative images of the proportions of CAR-positive cells in uninfected T cells and 123NL CAR-T cells detected by anti-CD34 antibody, IgG (H+L) antibody, anti-NKG2D antibody staining, and flow cytometry. c 123NL CAR-T cells with various dual-target CARs were cocultured with DAUDI, BJAB, and HMY2.CIR and THP1 cells were in equal proportions for 24 hours, and the cytotoxicity in each group was detected. d 123NL CAR-T cells with various dual-target CARs were cocultured with DAUDI, BJAB, and HMY2.CIR and THP1 cells were in equal proportions for 24 hours, and then the level of secreted IFN-γ (a cytotoxicity-related cytokine) in the medium was detected. Three independent repeated experiments were performed, and the data are expressed as the mean ± standard deviation; * $p < 0.05$, ** $p < 0.01$. MOCK indicates uninfected T cells, which were not involved in statistical analysis and are represented in gray).

cytometer. The results showed that 123NL CAR-T cells had target specificity (Figure 4b). The levels of related cytokines were significantly higher in the transduced cell lines than in the uninfected T cells, except for those in the DAUDI cells, which do not express CD123 or NKG2DLs (Figure 4c, d).

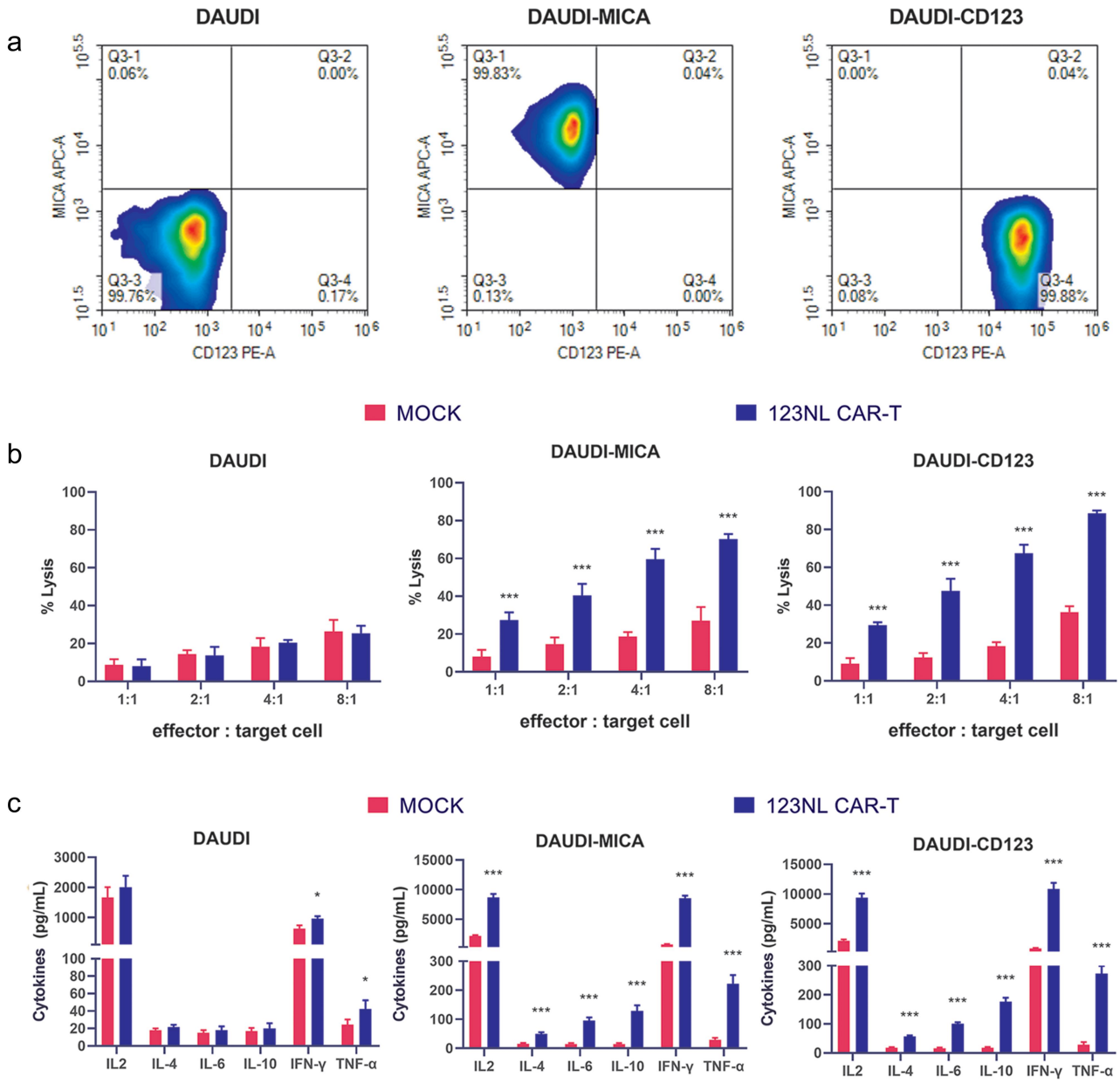

Figure 4. Target specificity analysis of 123NL CAR-T cells. a after staining with anti-MICA and anti-CD123 antibodies, DAUDI-MICA cells and DAUDI-CD123 cells (overexpressing MICA and CD123, respectively) were analyzed by flow cytometry with DAUDI cells as a reference. b 123NL CAR-T cells or uninfected T cells were cocultured with DAUDI, DAUDI-MICA, and DAUDI-CD123 cells at ratios of 1:1, 1:2, 1:4, and 1:8, and cytotoxicity was tested after 24 hours. c after 123NL CAR-T cells or uninfected T cells were cocultured with DAUDI, DAUDI-MICA, and DAUDI-CD123 cells at a ratio of 1:1 for 24 hours, the cytokines IL-2, IL-4, and IL-6 in the supernatant were detected. The levels of IL-6, IL-10, IFN-γ, and TNF-α were also detected. Three independent repeated experiments were performed, and the data are expressed as the mean ± standard deviation; * $p < 0.05$, ***$p < 0.001$. MOCK indicates uninfected T cells.

123NL CAR-T cells show antitumor activity in murine models

Next, we validated the in vitro results in mice. The research group selected 20 severely immunodeficient NSG mice and injected 2×10^6 cells in a luciferase-labeled tumor cell mixture (BJAB cells and HMY2.CIR cells were mixed 1:1; BJAB cells only express NKG2DLs, and HMY2.CIR cells only express CD123). To establish a murine tumor model, 20 mice were divided into 4 groups, 5 in each group. On the 5th day after tumor cell injection, each murine received 1×10^6 uninfected T cells, NKG2DLs single-target CAR-T cells, CD123 single-target CAR-T cells, or 123NL CAR-T cells. Thereafter, the tumor burden in the mice was monitored twice a week by intravital imaging technology (Figure 5a). Results showed that NKG2DLs single-target CAR-T cells and CD123 single-target CAR-T cells partially delayed tumor progression in mice compared to uninfected T cells, while mice treated with 123NL CAR-T cells had a lower tumor burden and their survival time was significantly longer (Figure 5b–d). Safety is an important consideration in the optimization of CAR-T cells. When the mice developed poor health or near death, we collected peripheral blood, detected relevant indicators of heart, liver, and

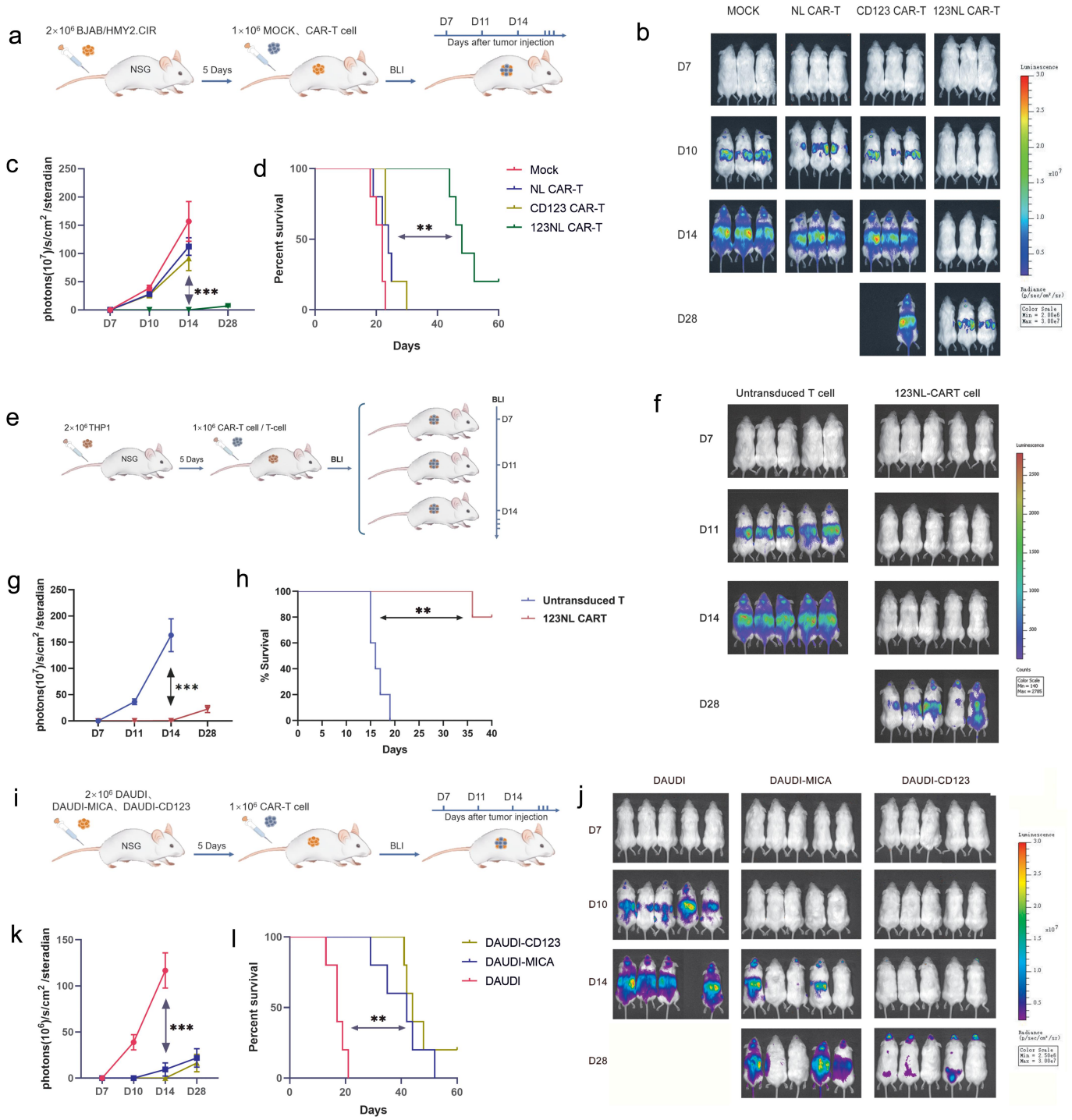

Figure 5. The effects of 123NL CAR-T cells in a murine model. a Experimental flow chart. The murine tumor model was established by tail vein injection of 2×10^6 luciferase-labeled tumor cells from a cell mixture (BJAB cells and HMY2.CIR cells at a 1:1 ratio). Five days after tail vein injection, uninfected T cells, NKG2DLs single-target CAR-T cells, CD123 single-target CAR-T cells, and 123NL CAR-T cells were administered. In vivo imaging technology was used to detect tumor burden in mice at planned monitoring time points. b at the planned monitoring time point, the tumor burden in each group of mice was detected (there were 5 mice in each group, and 3 representative images from each group of mice are shown). c Quantitative diagram of tumor burden in mice from each group. d the survival time curve of mice in each group. e flow chart of the experiment. In the experiments, 2×10^6 THP-1 cells labeled with luciferase were injected to establish a murine tumor model. There were five mice in each group. Five days after tumor cell injection, mice each received 1×10^6 123NL CAR-T or non-transduced T cells as treatment. Thereafter, in vivo tumor burden was monitored twice weekly and at planned monitoring time points by in vivo imaging. f tumor burden plots for each group of mice monitored at time points. g Quantitative plot of tumor burden in each group of mice. h survival time curves of mice in each group. i Experimental flow chart. For the experiment, 2×10^6 DAUDI, DAUDI-MICA, and DAUDI-CD123 cells labeled with luciferase were injected to establish murine tumor models. There were 5 mice in each group. Five days after tumor cell injection, the mice received 1×10^6 123NL CAR-T cells per murine as treatment. After that, the tumor burden in the mice was monitored twice a week by in vivo imaging technology, and the tumor burden in the mice was detected by in vivo imaging technology at the planned monitoring time point. j diagram of tumor burden in mice from each group at the planned monitoring time points. k Quantitative diagram of tumor burden in mice from each group. l survival time curves of mice from each group. Quantitative data are expressed as the mean ± standard deviation; ** $p < 0.1$. MOCK indicates uninfected T cells.

kidney function in the supernatant, separated the liver, spleen, and kidney tissues for HE staining, and analyzed the toxicity and side effects of the different CAR-T cells. Our results indicated that all mice were within normal limits for heart, liver and kidney related functional parameters, and HE staining showed no abnormal changes in the tissues (Fig. S_4a-e). These results demonstrate that 123NL CAR-T cells have target specificity and can kill cells expressing NKG2DLs or CD123 in mice, and no toxic side effects were found that directly affected murine heart-, liver-, or kidney-related functions or tissue. In addition, we used THP-1 tumor cells to establish a mouse tumor model with co-expression of both target antigens. Compared with non-transduced T cells, 123NL CAR-T cells significantly reduced tumor burden and prolonged survival time of mice (Figure 5e–h). For further demonstration of the in vivo targeting specificity of 123NL CAR-T cells, we again selected 15 severely immunodeficient NSG mice and injected 2×10^6 fluorescein-labeled DAUDI, DAUDI-MICA and DAUDI-CD123 cells via tail vein for establishing mouse tumor models, 5 in each group. On the 5th day after tumor cell injection, the mice received 1×10^6 123NL CAR-T cells per murine as treatment. After that, they were monitored twice a week by in vivo imaging technology (Figure 5i). It showed that compared with mice that received uninfected T cells, mice treated with 123NL CAR-T cells had a lower tumor burden and longer survival time (Figure 5j–l).

123NL CAR-T cells killed primary tumor cells and immunosuppressive cells, and their cytotoxicity can be terminated

The above research results suggest that 123NL CAR-T cells have an excellent cytotoxicity and target specificity. We collected bone marrow samples from 8 AML patients whose tumors, M-MDSCs, and M2 cells all expressed (over 50%) CD123 or NKG2DLs, and the extracted mononuclear cells were incubated with 123NL CAR-T cells or uninfected T cells. Compared with uninfected T cells, 123NL CAR-T cells showed significant cytotoxicity on tumor cells, M-MDSCs, and M2 cells (Figure 6a–c). After co-incubation, the cells were subjected to a colony culture experiment to analyze the effect of 123NL CAR-T cells on normal bone marrow hematopoiesis. To evaluate the safety of 123NL CAR-T cells more accurately, we collected samples from 3 B-ALL patients with as similar of a tumor burden as possible and incubated them with CD19 CAR-T cells. The colony culture experiment was performed under the same conditions. The results showed that compared with the uninfected T cells, the 123NL CAR-T cells and CD19 CAR-T cells mainly affected myeloid hematopoiesis and had less effect on erythroid hematopoiesis. Compared with the CD19 CAR-T cells, the 123NL CAR-T cells inhibited myeloid colony formation more severely (Figure 6d). These results may indicate that bridging hematopoietic stem cell transplantation may be needed in the clinical application of 123NL CART cells. To verify the suicide function of the RQR8 structure in 123NL CAR-T cells, different concentrations of rituximab were incubated with the 123NL CAR-T cells, uninfected T cells, and AML patient bone marrow mononuclear cells (BMMCs). Rituximab at a dose of less than 10 μg/ml had little effect on these types of cells (Figure 6e). Next, we added 10 μg/ml rituximab to AML patient BMMCs and 123NL CAR-T cells or uninfected T cells. The BMMCs have NK cells and tumor cells expressing CD123 and NKG2DLs, which can be involved in antibody-dependent cellular cytotoxicity and CAR-T cell-related cytotoxicity, respectively. Results showed that compared with its effects on the uninfected T cells, rituximab significantly killed the 123NL CAR-T cells and inhibited their cytotoxicity and related cytokine secretion (Figure 6f–h). These results preliminarily prove that the RQR8 structural protein on the surface of 123NL CAR-T cells can have a suicidal function.

Discussion

There have been many studies of treating AML with CAR-T cells, which include CAR-T cells targeting CD33, CD123, CLL-1, NKG2DLs, and other antigens.[46,47] The results of existing clinical studies have shown that although CAR-T cells targeting CD33, CD123, and CLL-1 have certain curative effects, they face problems such as poor persistence, insufficient efficacy, and target escape.[48,49] Dual-targeted CAR-T cells have been found in studies to have more activity and affinity for tumor cells than single-targeted CART cells, and more importantly, dual-targeted CART cells control disease more consistently.[50–53] CD123 and NKG2DLs are good CAR targets as they are known to be elevated in AML cells. And both our data and a large number of other research results indicate that CD123 and NKG2DLs are expressed in M-MDSCs and M2 cells in the tumor microenvironment. To avoid antigen-negative recurrence because of antigen escape and antigen-positive recurrence caused by the inhibition of CAR-T cell function and persistence by the tumor immune microenvironment, we developed a 123NL dual-target CAR that simultaneously targets tumors and immunosuppressive cells as a more comprehensive disease treatment strategy.

First, to demonstrate the feasibility of simultaneously targeting CD123 and NKG2DLs, we collected bone marrow specimens from patients and healthy donors. We found that most of the patient tumor cells, M-MDSCs, and M2 cells expressed CD123, while NKG2DLs expression was low in M-MDSCs and tumor cells but not in M2 cells. Previous studies have shown that NKG2DLs are under-expressed in primary AML cells, and the addition of IFN-γ and other cytokines can promote its significant upregulation.[43] In this study, similar results were not obtained. There have also been reports that the demethylating drug decitabine can increase the expression of NKG2DLs,[27] but this was not performed in our study. However, the low level of NKG2DLs expression in AML cannot explain the clinical efficacy of NKG2DLs CAR-T cells. There may be other indirect mechanisms affecting AML cells.

In our research, we found that NKG2DLs CAR-T cells have fratricide killing, especially when CD28 is used as a costimulatory or transmembrane domain. The use of 41BB costimulatory and CD8 hinge structures can reduce this effect. This result may also be one of the reasons why most of the NKG2DLs CAR-T cells in the current study featured 41BB

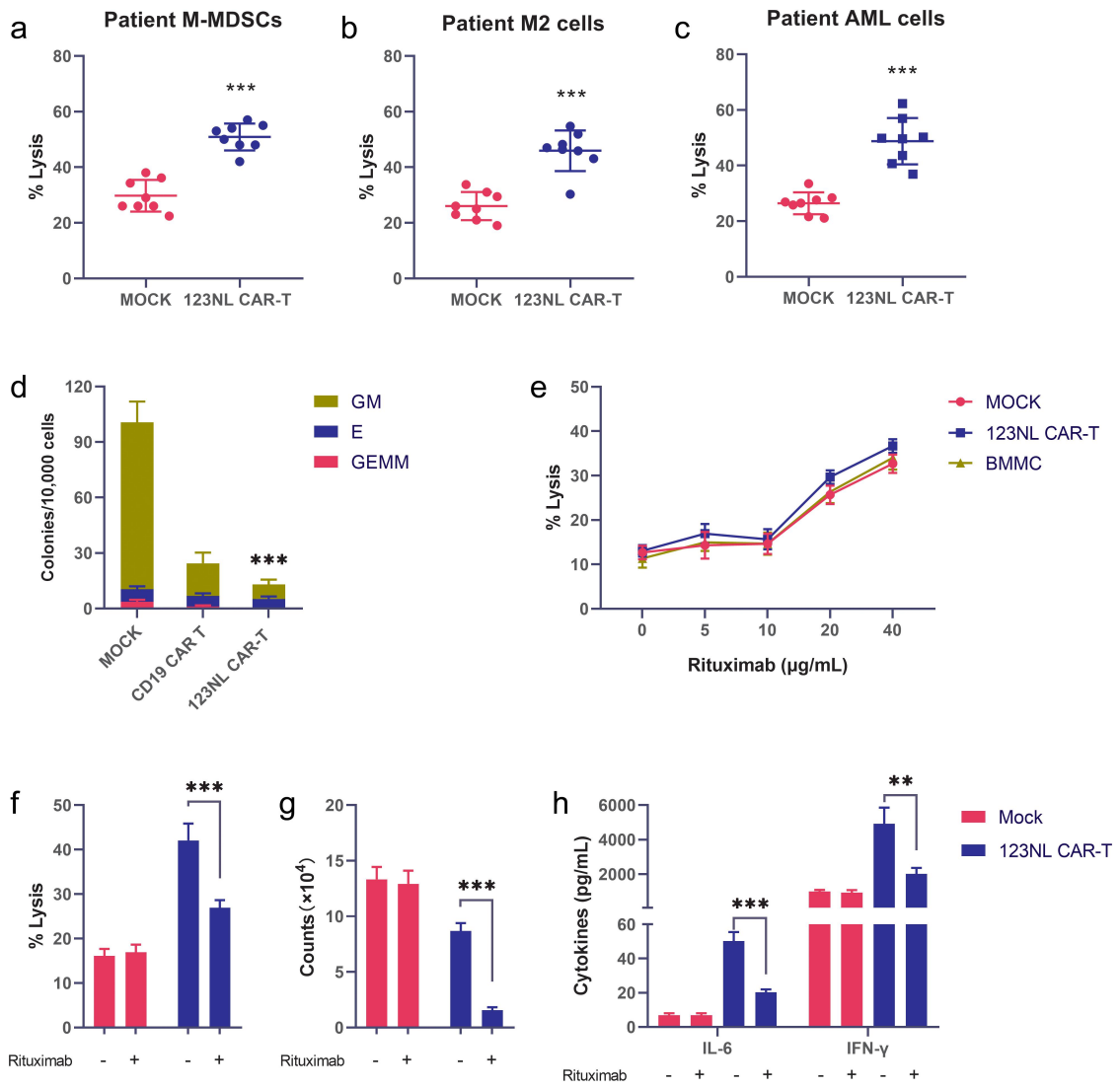

Figure 6. 123NL CAR-T cells have functions in primary cells and their toxicity is controllable. a-c 123NL CAR-T cells or uninfected T cells were cocultured with BMMCs from AML patients for 24 hours, and their cytotoxic effects on M-MDSCs, M2 cells, and tumor cells in primary bone marrow cells from AML patients were tested. d 123NL CAR-T cells, CD19 CAR-T cells, and uninfected T cells were cocultured with primary bone marrow cells for 24 hours, and then colony culture experiments were performed. After 2 weeks, the numbers of various colonies were counted. GM is the granulocyte-macrophage colony-forming unit, E is the red blood cell colony-forming unit, and GEMM is the granulocyte, red blood cell, macrophage, and megakaryocyte colony-forming unit. e different concentrations of rituximab were incubated with 123NL CAR-T cells, uninfected T cells, and BMMCs from AML patients. Cytotoxicity was detected 24 hours later. f and g a total of 1×10^5 123NL CAR-T cells or uninfected T cells and BMMCs from AML patients were cocultured with or without 10 μg/ml rituximab at a ratio of 1:1 for 24 hours. Tumor cell cytotoxicity and the number of 123NL CAR-T cells or uninfected T cells were detected. h a total of 1×10^5 123NL CAR-T cells or uninfected T cells and BMMCs from AML patients were cocultured at a ratio of 1:1 with or without 10 μg/mL rituximab for 24 hours, and the levels of the cytokines IL-6 and IFN-γ in the supernatant were detected. Three independent repeated experiments were performed, and the data are expressed as the mean ± standard deviation; $^{**}p < 0.01$, $^{***}p < 0.001$. MOCK indicates uninfected T cells.

costimulatory and CD8 hinge structures.[22–27] We designed a series of bicistronic CARs by optimizing the hinge and costimulatory domains in the anti-CD123 CAR structure. Within these CARs, BCAR3 has the highest proportion of naïve cells while the level of immune checkpoint molecules is lower. There have been a large number of reports showing that CAR-T cells with a higher proportion of cells with a naive phenotype and lower levels of immune checkpoint molecules have better persistence and better efficacy.[43–45] Therefore, we used 123NL CAR-T cells with the BCAR3 structure for our experiments. Although CD19 CAR-T cell products using CD28 costimulatory domains did not show advantages in terms of the proportion of cells with a naive phenotype and the levels of immune checkpoint molecules, they have obtained better results in clinical studies in the treatment of lymphoma. Due to labor and financial limitations, we selected only one of the CAR structures for detailed research, and a more comprehensive comparison may result in a better structure.

We demonstrated the good target specificity of 123NL CAR-T cells in vitro and in vivo and a good safety profile. In addition, we collected primary bone marrow cells from AML patients and confirmed the cytotoxic effects of 123NL CAR-T cells on tumor cells and immunosuppressive cells (M-MDSCs and M2 cells). To validate the in vitro results using primary cells in an in vivo setting, we attempted to establish a mouse model using primary cells. Despite utilizing highly immunodeficient NSG mice and further reducing immunity through re-irradiation before cell implantation, the injected cells were

swiftly eliminated. As a result, we were unable to obtain entirely reliable data. Moving forward, our focus will be on identifying a more suitable research model.

A major concern in translating our 123NL CAR-T cells to the clinic is the potential for "toxicity" effects. We adopted an approach of rituximab binding to RQR8 protein and therefore depleting CAR-T cells for the safe clinical usage of CAR-T cells. Verification of the RQR8 suicide switch requires the presence of NK cells or the complement system. We collected primary mononuclear cells from patients and conducted preliminary verification. Rituximab did not completely eliminate 123NL CAR-T cells due to insufficient NK cell activity, but obvious suicidal effects were observed. In addition, we did not use mice with immune functions for the in vivo experiments, so the suicide effect could not be verified in vivo, and a more complete model may be needed in the future for verification. However, several clinical studies have used the RQR8 suicide switch, supporting the rationality of our 123NL CAR structure design.[54–56]

Despite previous studies demonstrating the favorable safety profile of targeting CD123 and NKG2DLs,[18–27] it is important to note that these studies primarily focused on single-target CAR-T cells. While single-target CAR-T cells have been tested in clinical trials, the safety of dual-target CAR-T cells still needs to be specifically demonstrated. Further research and clinical studies are required to assess the safety and potential side effects associated with the use of dual-target CAR-T cells. In our study, we conducted an examination of histological images and measured biochemical indicators in mice following treatment with 123NL CAR-T cells to provide evidence of its safety. Cytokine release syndrome is a significant concern in CAR-T cell therapy. In our study, in vivo cytokine profiling was not conducted. Furthermore, due to the low abundance of CAR-T cells in the peripheral blood of mice, we were unable to obtain kinetic data on CAR-T cells in mice. This limitation should be acknowledged in the study.

In summary, 123NL CAR-T cells present a promising opportunity for the clinical treatment of AML. Some AML patients experience relapse shortly after receiving CAR-T therapy, preventing them from proceeding to the next stage of treatment. Our study demonstrates that 123NL CAR-T cells exhibit stronger anti-AML effects compared to single-target CARs. 123NL CAR-T therapy may serve as a valuable bridge for AML patients to undergo treatments like hematopoietic stem cell transplantation. Moreover, it may offer extended survival for patients who do not meet the criteria for additional therapy. These findings highlight the potential of dual-targeted CAR-T cells as a unique and powerful approach to enhance current AML treatments.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Authors' contributions

XJ and MFZ designed the research. XJ, DNX, RS, XX, MZ, JXW, YBY, XYH, and JXM performed the research. XJ, WYL, and MFZ analyzed the data. XJ wrote the manuscript. XJ, DNX, RS and MFZ revised the manuscript.

Availability of data and materials

All data obtained and/or analyzed during the current study are available from the corresponding authors upon reasonable request.

Abbreviations

CAR-T	Chimeric antigen receptor-engineered T cells
AML	Acute myeloid leukemia
M-MDSCs	Monocyte-like myeloid-derived suppressor cells
M2 cells	Alternately activated macrophages
NKG2DLs	NKG2D ligands
123NL CAR-T	Dual-target CAR-T cells targeting CD123 and NKG2DLs
B-ALL	B cell acute lymphoblastic leukemia
LFA-1	Lymphocyte function-associated antigen 1
BMMCs	Bone marrow mononuclear cells

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Tianjin First Central Hospital. All patients signed an informed consent form. The animal experiment protocol was approved by the Animal Care and Use Committee of Tianjin Medical University.

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